United States Army Medical Research Institute of Infectious Diseases

Increased Stability of a Designed, Single Domain Ricin A-chain Vaccine

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Goal: Create a soluble and stable platform for safe presentation of a neutralizing epitope against ricin A-chain.

Limitations of earlier vaccine candidates:

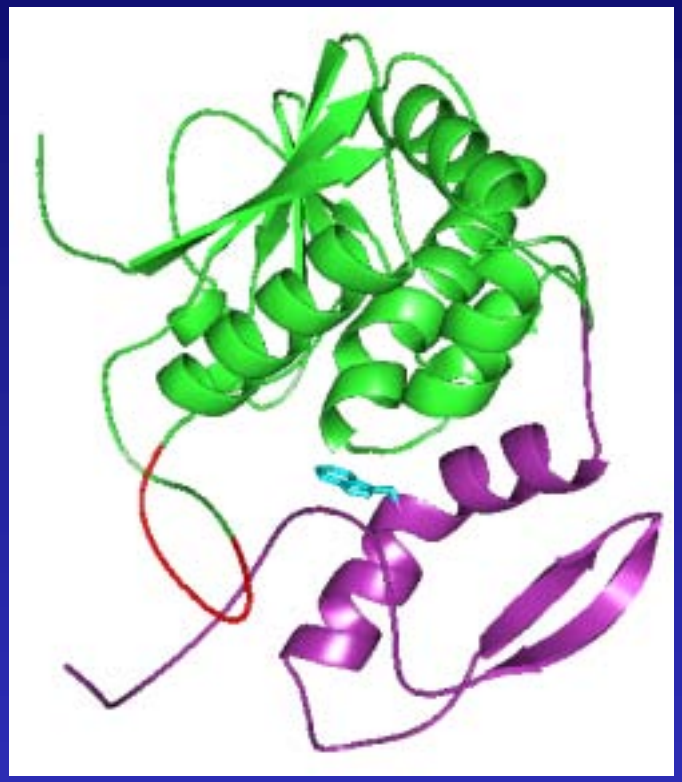
- Residual catalytic activity
- Poor solubility -- aggregation
- Relatively unstable

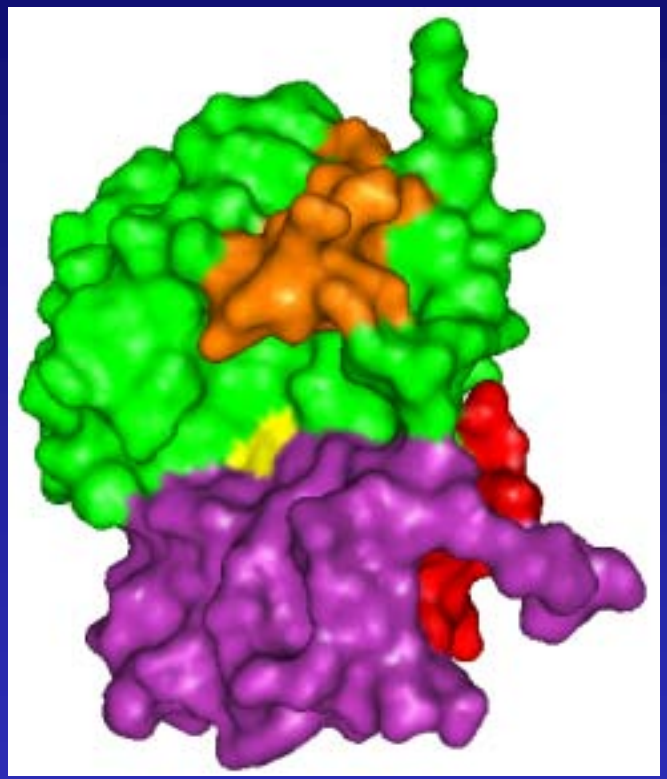
Solution: Design a stable, monomeric, single-domain derivative of RTA by protein engineering.

• Mark Olson	modeling
• Virginia Roxas	cloning
• Leonard Smith, Robert Wannemacher	toxicology, immunology
• many others … team effort	

The A-chain of Ricin (267 a.a.)

(1rtc.pdb, Mlsna et al., 1993, *Protein Sci. 2*:429-435)





Vaccine candidates created: RTA 1-33/44-198

RTA 1-198

Design considerations:

1. Conservation of structure from WT RTA
   - Maintain epitope in natural conformation.

2. Soluble and monomeric
   - No aggregation.

3. High stability
   - Avoid degradation during production and storage.
   - Avoid need for stabilizing excipients in formulation, the "cold chain."

4. No toxicity, adequate neutralizing antigenicity
   - *Verified in mouse model*

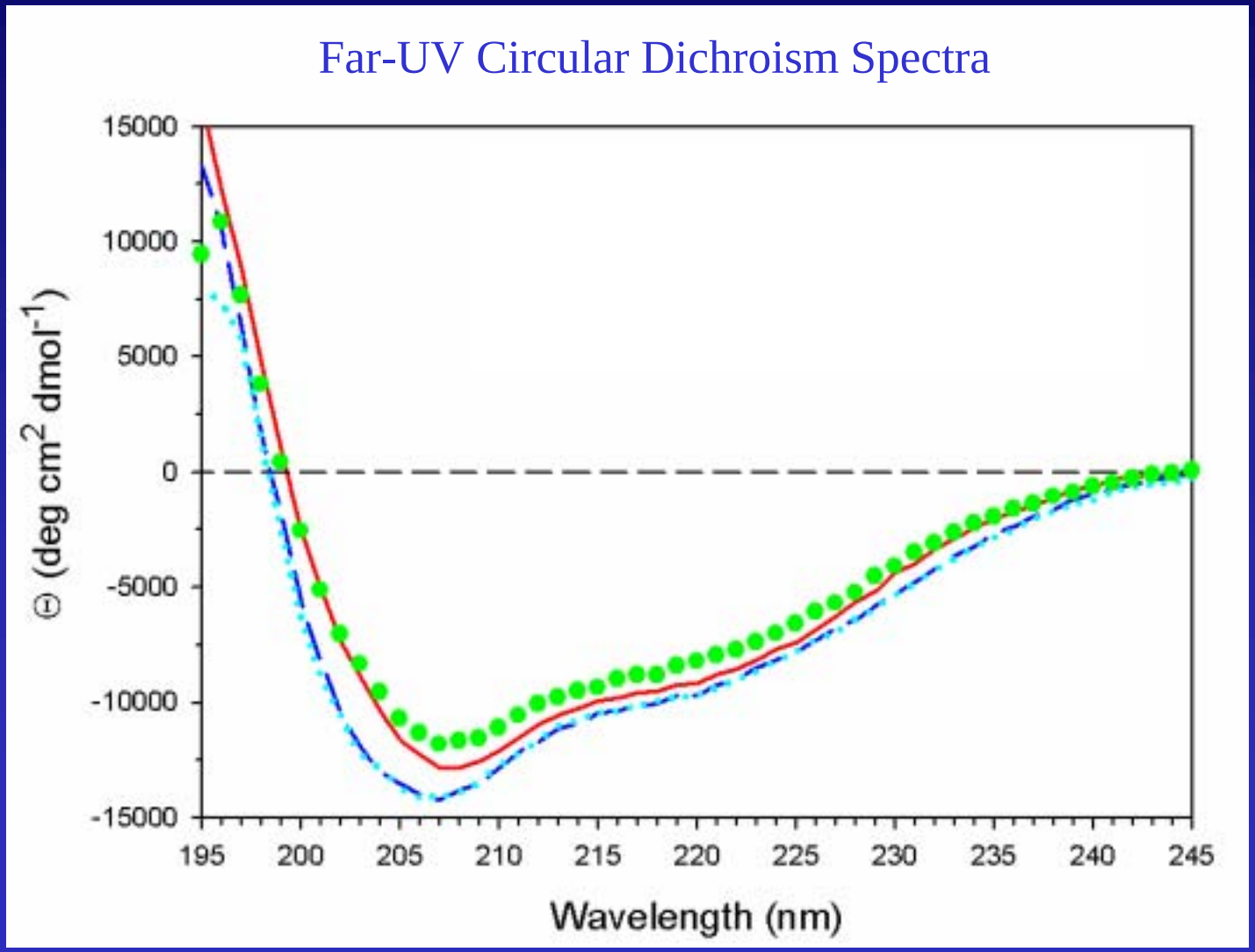
Secondary structure contents close to that of RTA
are predicted for the mutants,
if the N-terminal domain is not altered by the residue deletions

Table 1. Predicted Secondary Structure Content[a]

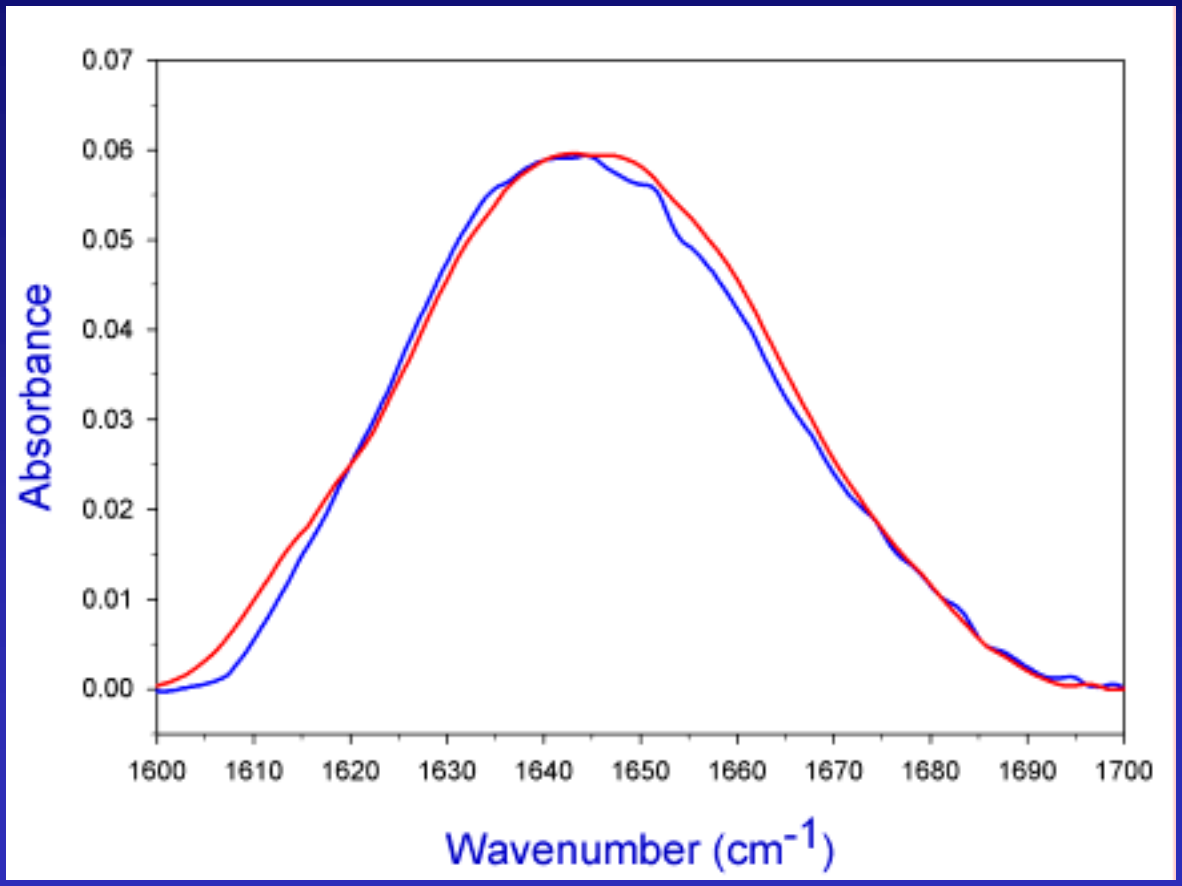
Protein	% β sheet	% α helix	% Other
RTA	16	32	52
RTA 1-198	16	34	50
RTA 1-33/44-198	17	36	47

[a] Percentage of amino acids predicted in each type of secondary structure, based on the known structure of RTA (Mlsna et al., 1993).

The RTA derivatives are folded and have secondary structure contents similar to recombinant and natural wild-type RTA



Fourier-transform infrared spectra in the amide I' region also indicate conservation of structure



rRTA

1-33/44-198

RTA 1-33/44-198 is a monodisperse monomer in solution

Table 2. Dynamic Light Scattering Measurements

Protein	R_h[a]	hydrodynamic MW_r[b]	actual MW_r[c]
RTA	2.53	29.5×10^3	30.0×10^3
RTA 1-198	2.16	20.4×10^3	22.5×10^3
RTA 1-33/44-198	2.11	19.3×10^3	21.3×10^3

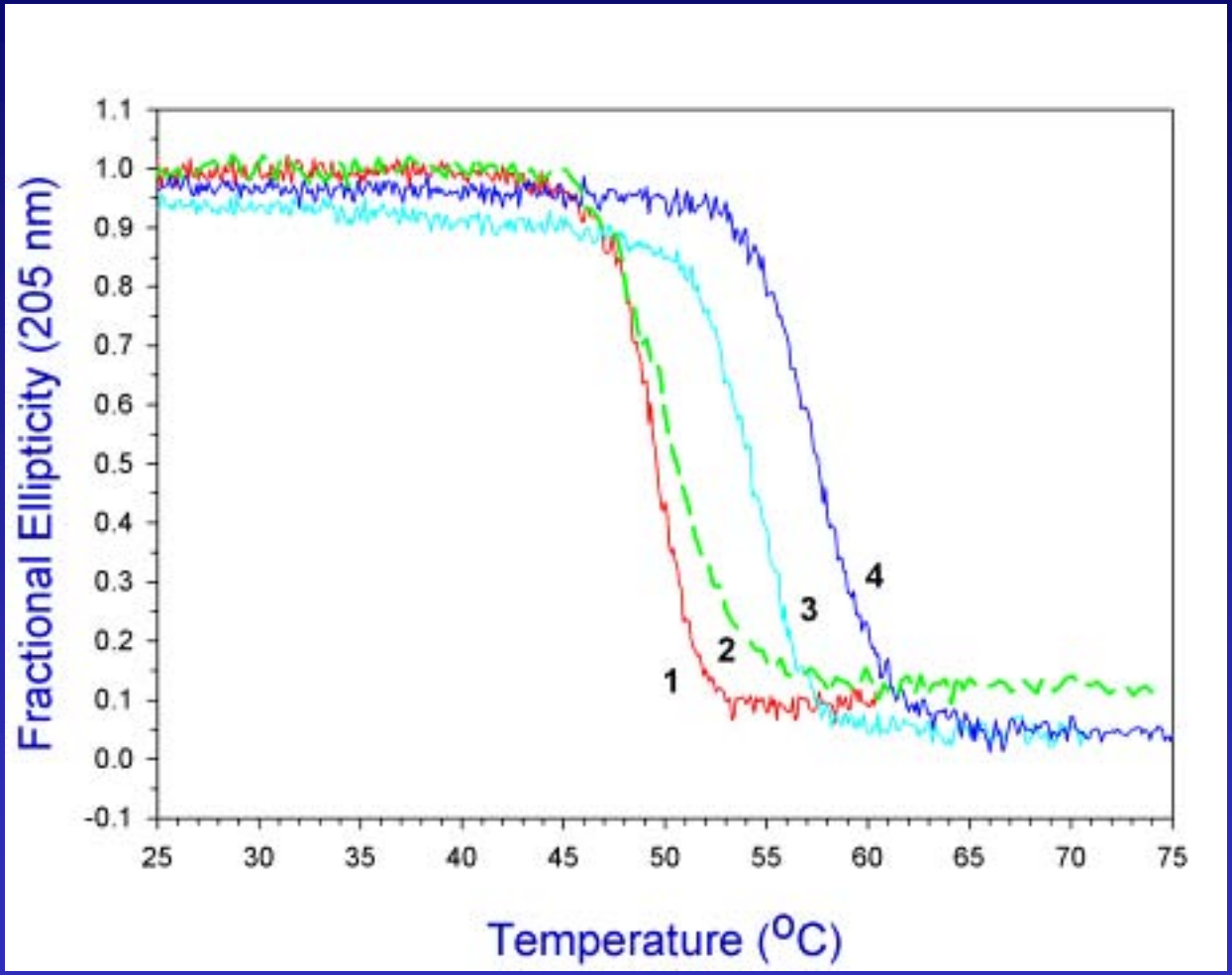
[a] The hydrodynamic radius of the protein in solution as measured by DLS, in nanometers.

[b] Molecular weight calculated using the Dynamics software (Protein Solutions).

[c] Molecular weight predicted from the amino acid sequence.

0.8 mg/ml protein in PBS buffer
PBS buffer was used in all experiments because this is the expected formulation condition.

RTA 1-33/44-198 and RTA 1-198 have increased thermostability



1 rRTA

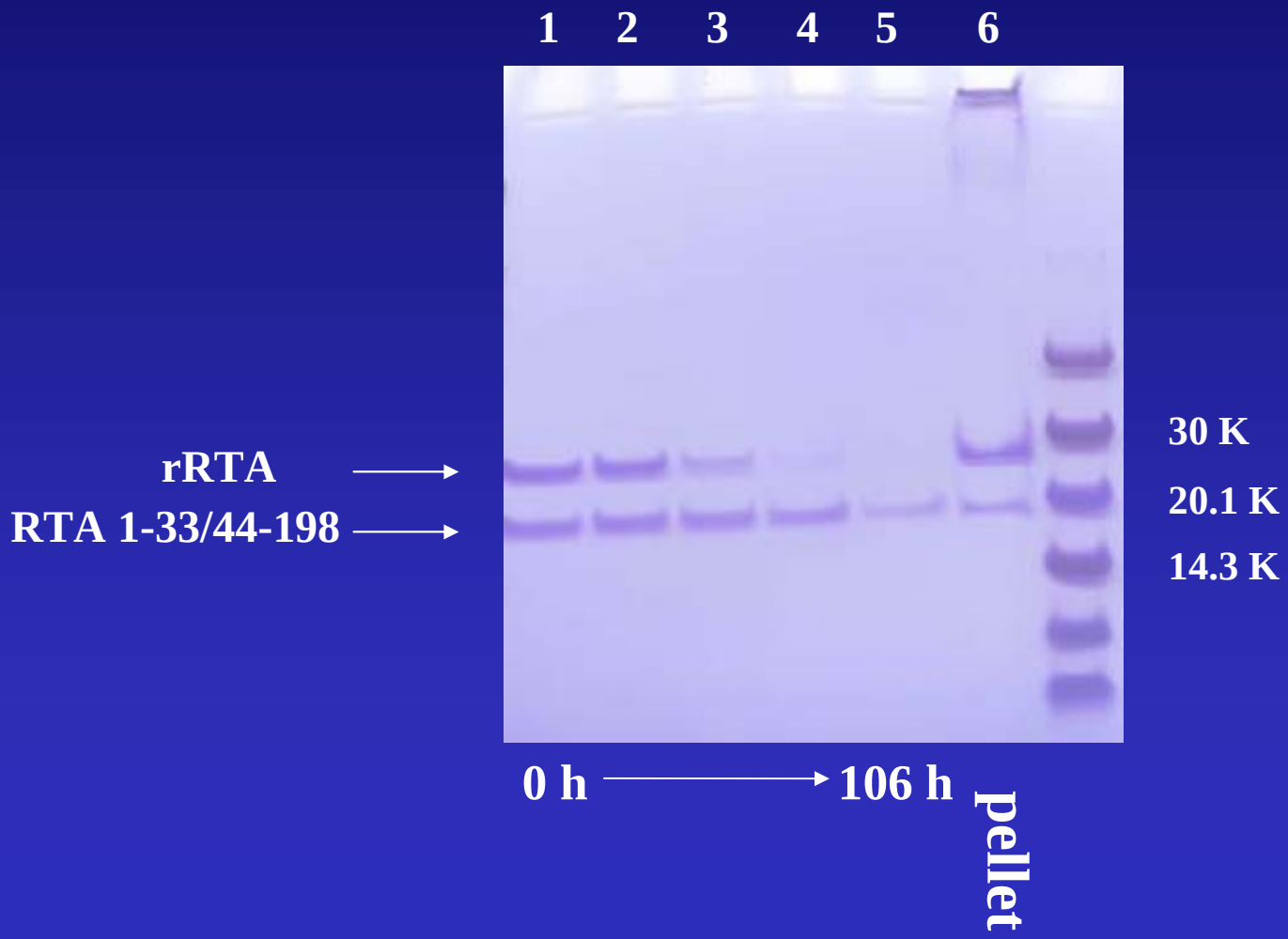
2 nRTA

3 1-198

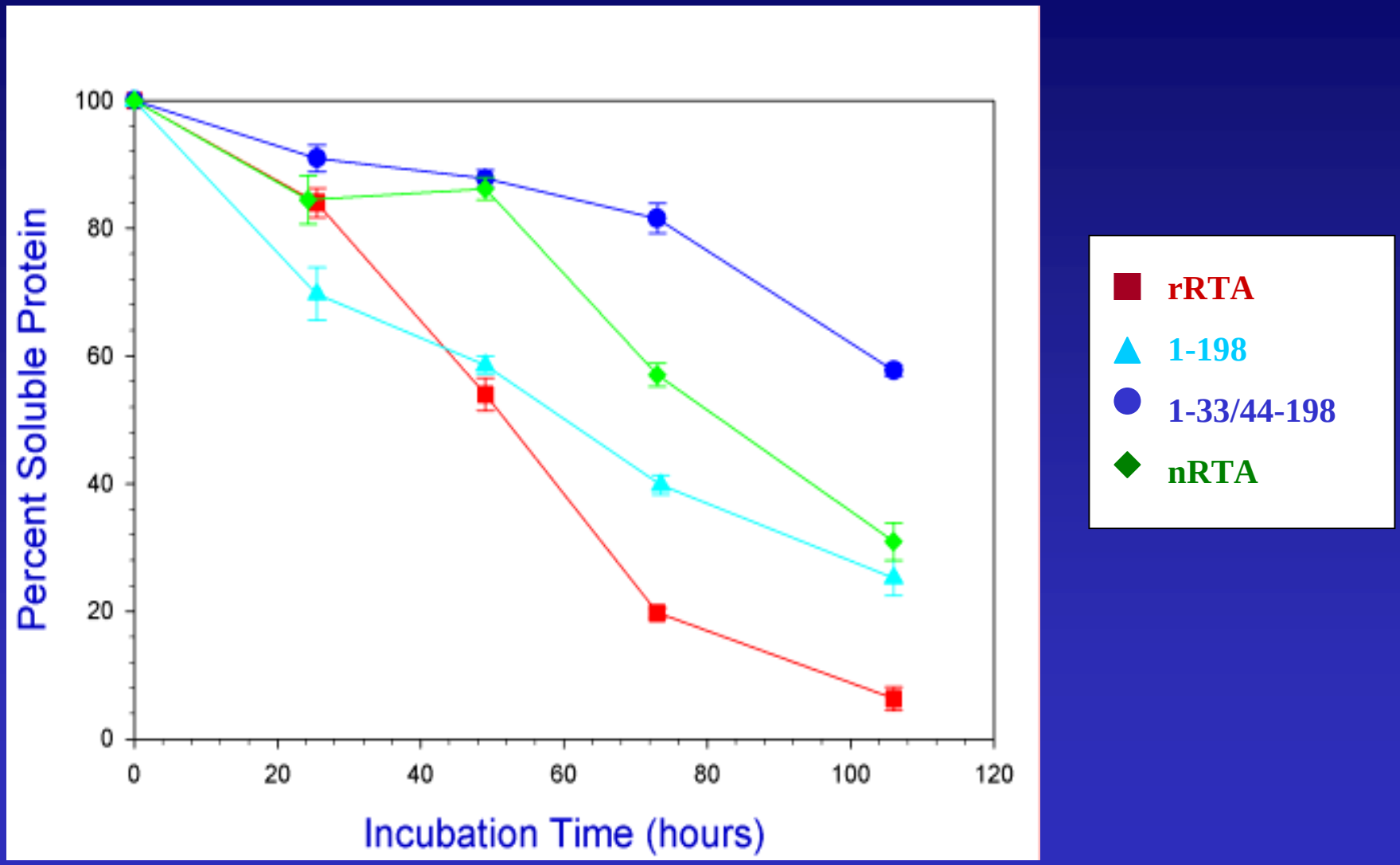
4 1-33/44-198

RTA 1-33/44-198 is more stable to long-term incubation at 37 °C

rRTA and RTA 1-33/44-198 were mixed together and incubated at 37 °C for a period of time, then precipitated protein was removed by centrifugation and the soluble fraction run on a gel.

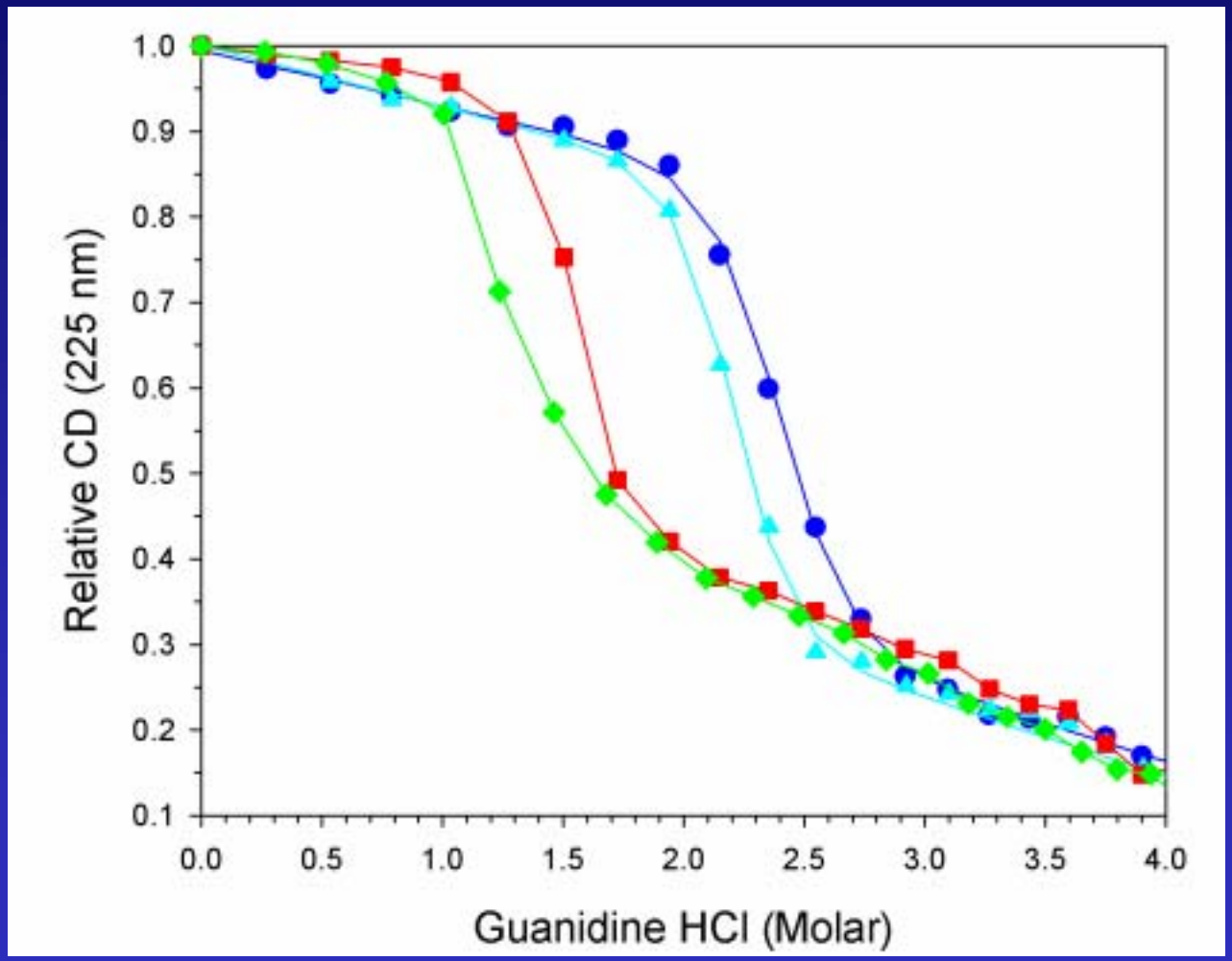


% Solubility of proteins after extended incubation at 37 °C
RTA 1-33/44-198 remains in solution longest



Denaturation by guanidine-HCl followed by CD at 225 nm

RTA 1-33/44-198 is the most stable to chemical denaturation



Stabilities of wild-type and mutant RTA molecules

Table 3. Stability parameters of RTA molecules.

Protein	T_d [a]	C_m [b]	% soluble [c]
rRTA	46.6 ±0.1	1.56 ±0.022	6
rRTA 1-198	50.8 ±0.5	2.29 ±0.004	25
rRTA 1-33/44-198	53.1 ±0.1	2.53 ±0.041	58
nRTA	45.6 ±0.1	1.39 ±0.020	31

[a] Temperature of onset of denaturation in thermal melting in °C as monitored by CD at 205 nm. T_d was taken as the point at which the signal had diverged from that of the native state by 5%. Results are the average and standard error of three measurements.

[b] The midpoint of denaturation in Molar [GuHCl] as measured by CD at 225 nm.

[c] The percent of protein remaining soluble after incubation at 37 °C for 106 hours. Errors are shown in Figure 5.

Demonstrated:

1. Conservation of secondary structure from wild-type RTA
   - Circular dichroism and FTIR data

2. Soluble and monomeric
   - Dynamic light scattering

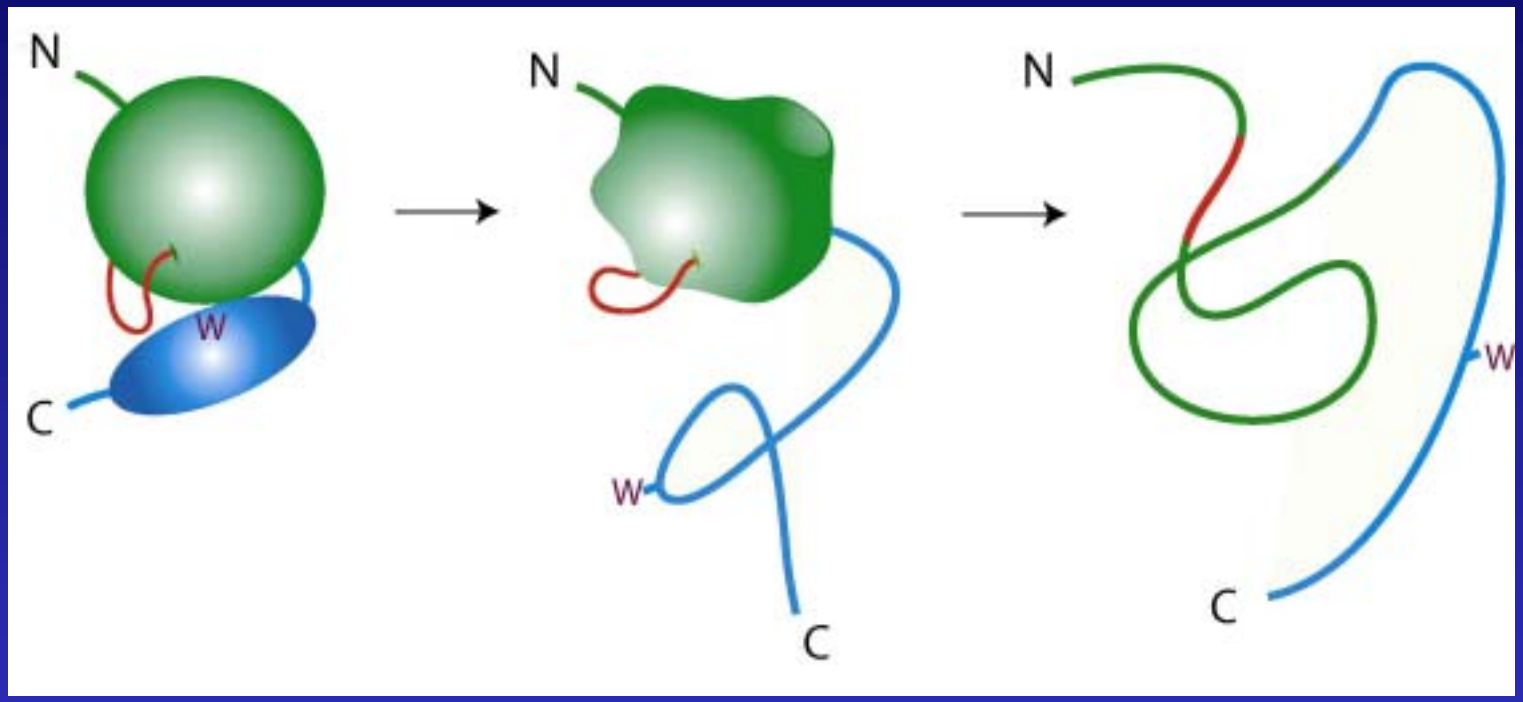
3. High stability
   - Melting curves
   - Extended incubation at physiological temperature

What is the origin of the increased stability of RTA 1-33/44-198 and 1-198?

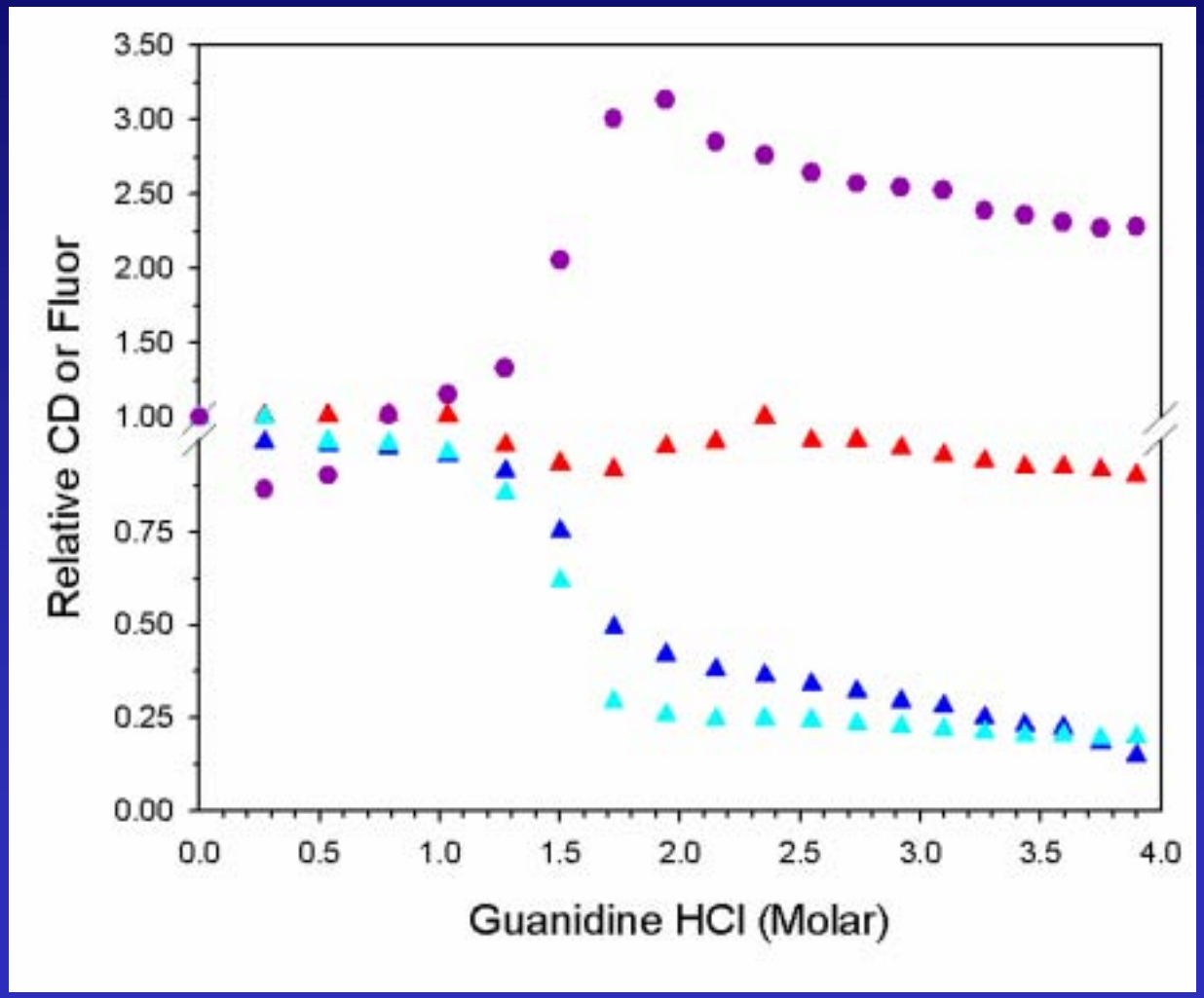
Three-state unfolding of RTA through an intermediate

12 Tyr

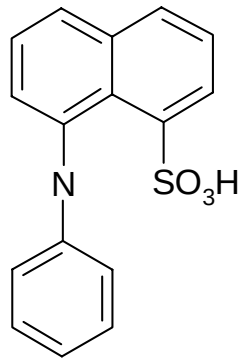
1 Trp
2 Tyr



Denaturation of wild-type rRTA by guanidine-HCl proceeds through a partially unfolded intermediate

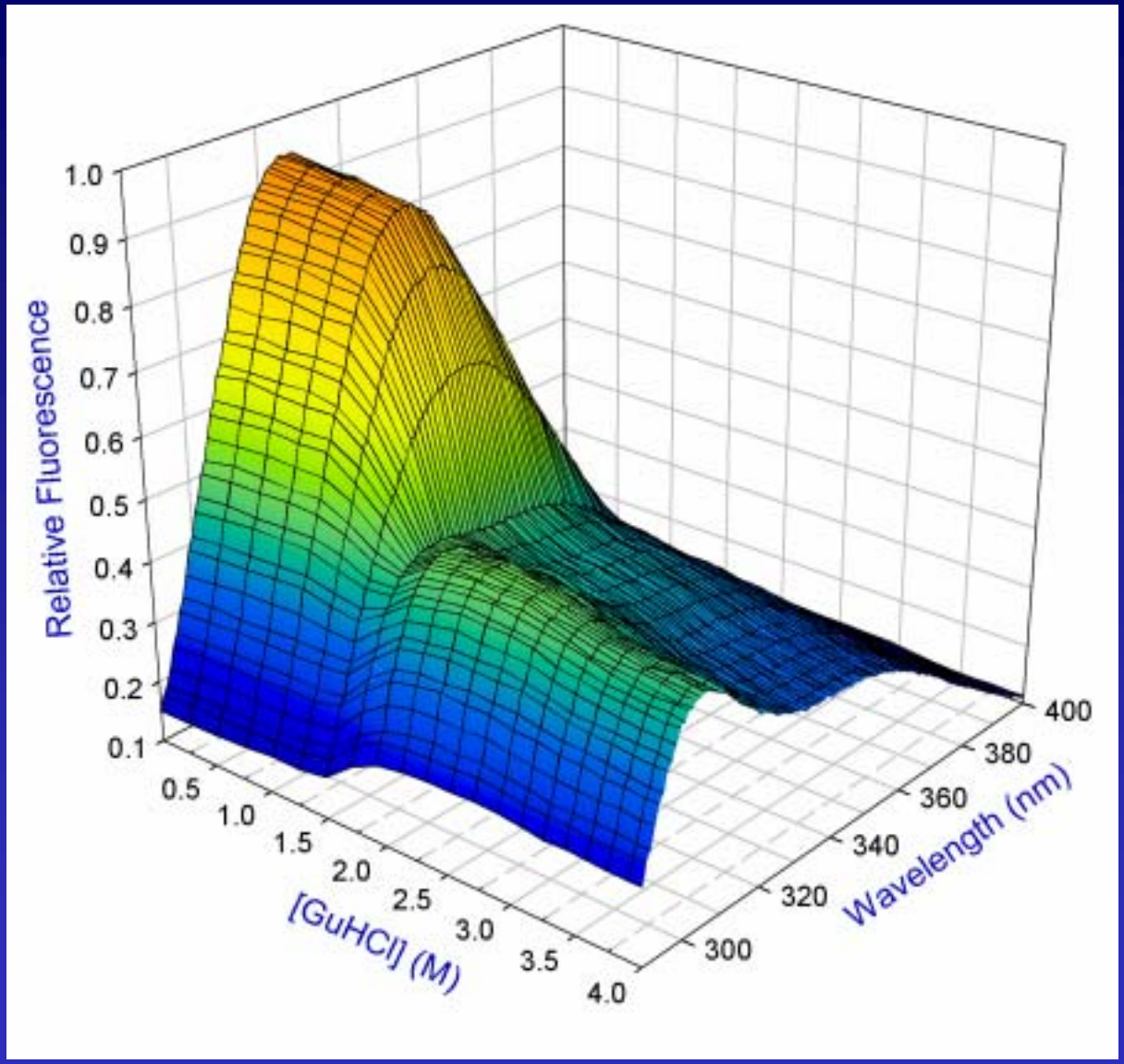


- Fluor ex 295 em 320
- CD 225 nm
- Fluor ex 280 em 303
- ANS Fluor 480 nm



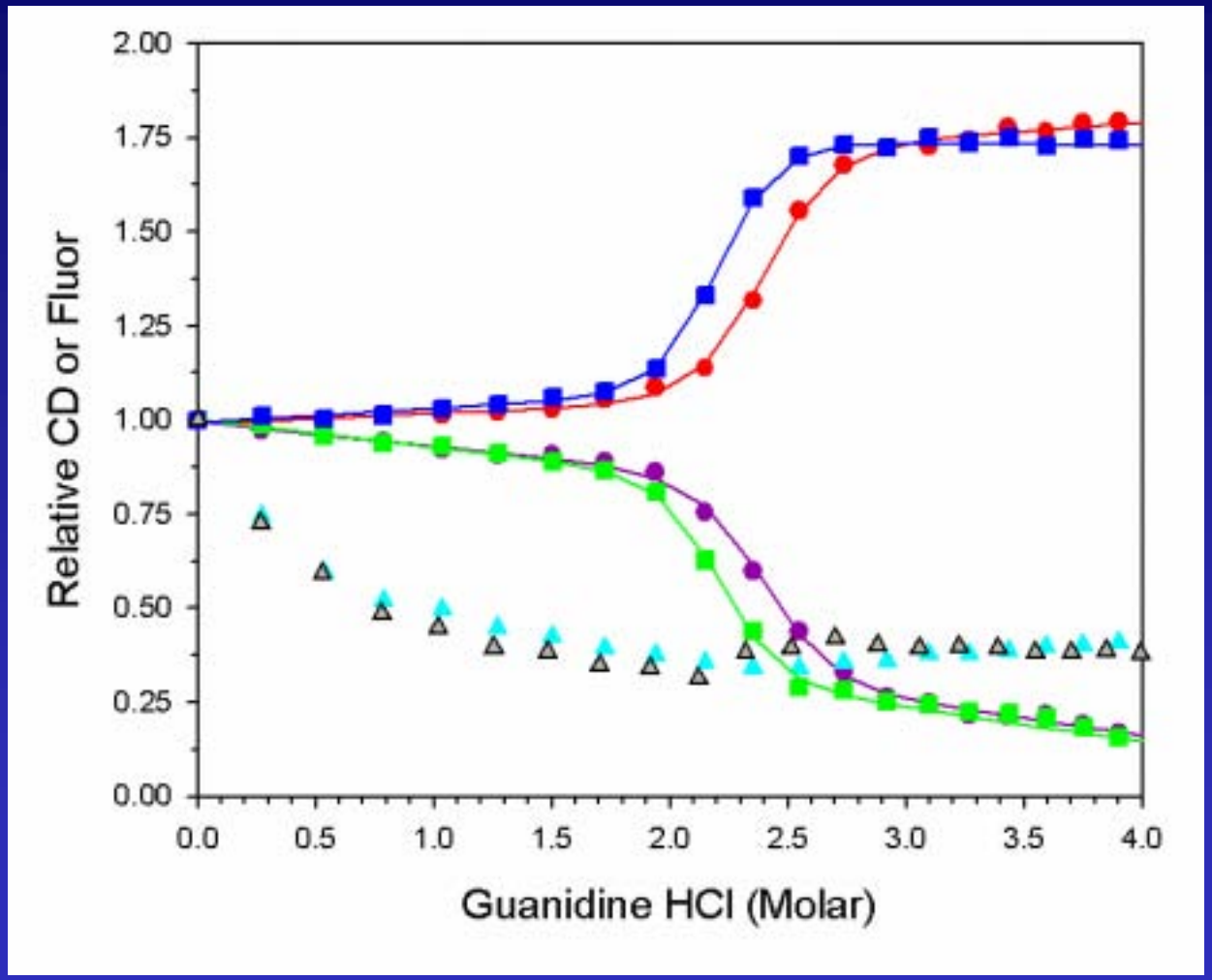
Anilinonaphthalene sulfonic acid (ANS)

Denaturation of wild-type rRTA by guanidine-HCl



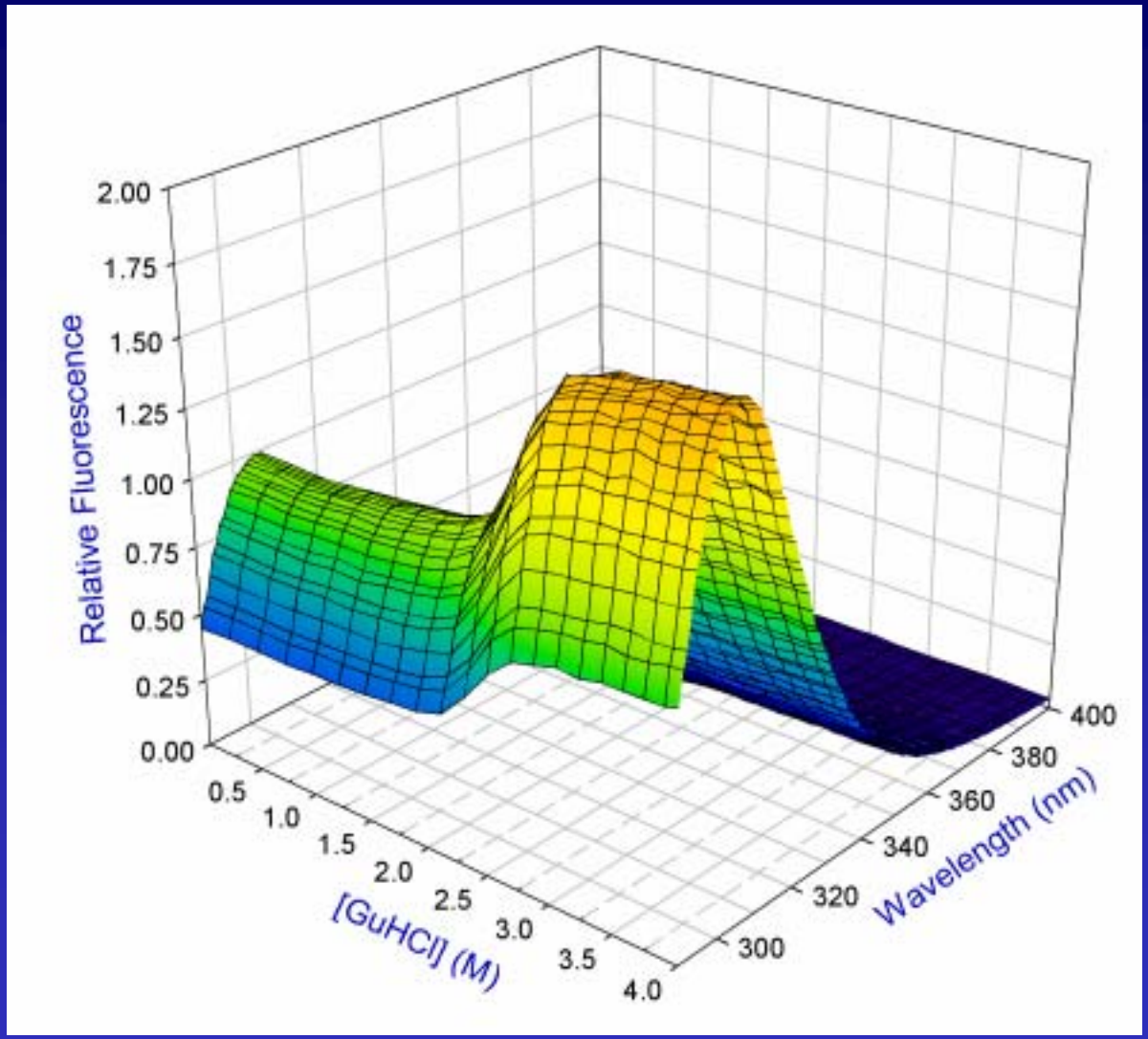
Followed by fluorescence emission with excitation at 280 nm

Denaturation of the truncated RTA derivatives by guanidine-HCl shows no intermediate -- a two-state process



- 1-33/44-198 CD 225
- 1-198 CD 225
- 1-33/44-198 Fluor (ex 280, em 303)
- 1-198 Fluor
- 1-33/44-198 ANS
- 1-198 ANS

Denaturation of RTA1-33/44-198 by guanidine-HCl

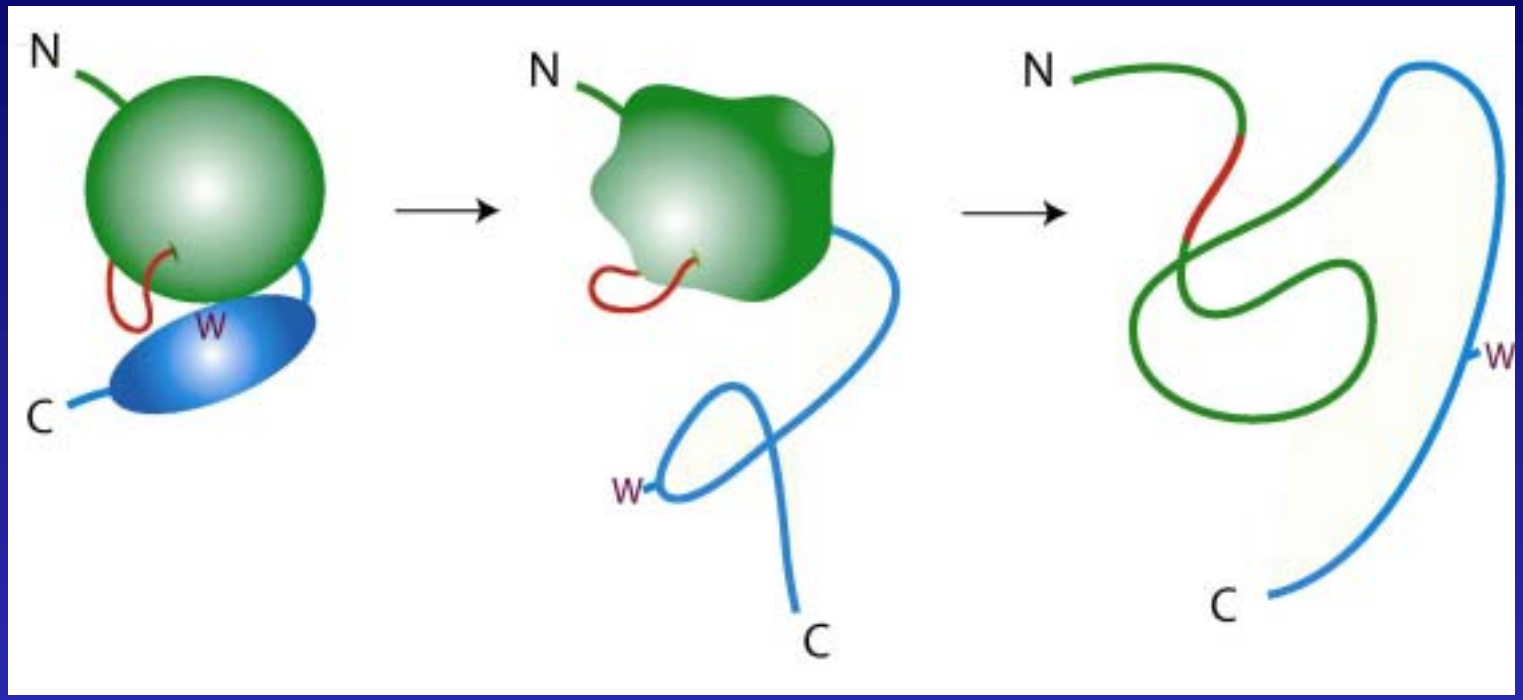


Followed by tyrosine fluorescence emission with excitation at 280 nm

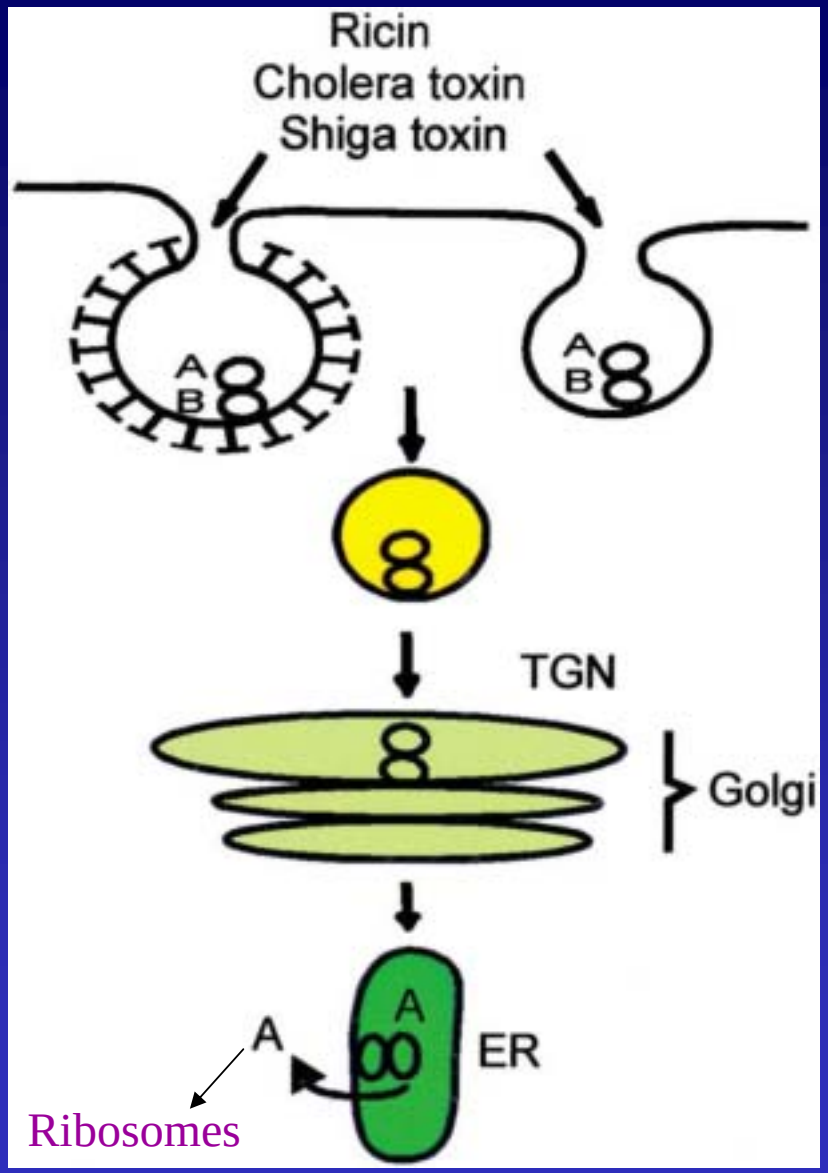
Three-state unfolding of RTA through an intermediate

12 Tyr

1 Trp
2 Tyr



Comparison of tryptophan vs. tyrosine fluorescence emission data and the absence of the intermediate for the vaccine candidates indicate that the C-terminal domain of RTA is relatively unstable, unfolding before the N-terminal domain.



Intracellular translocation of RTA uses pathways for export from the ER of misfolded proteins.

-- A physiological role for an intermediate state?

[Argent et al., 2000, *JBC 275*:9263-9269, Day et al., 2002, *Biochemistry 41*:2836-2843, Sandvig and van Deurs, 2002, *FEBS Letts. 529*:49-53]

Main conclusions

1. The RTA vaccine candidates are soluble, folded, monomeric, and more stable than WT RTA.
   - Superior biophysical properties for a vaccine.

2. Unfolding of RTA involves a partially unfolded intermediate state with a disrupted C-terminal domain.

3. Mutant derivatives lacking the C-domain do not adopt the intermediate state, which helps explain their greater stability.
   The native state of RTA is limited in its stability by a propensity to transform to a partially unfolded state.

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